

Application No. 10/664,468  
Amendment dated June 10, 2010  
After Final Office Action of January 21, 2010

Docket No.: 31174/30016A

# **EXHIBIT “B”**

## Anti-angiogenic Property of Edible Berries

SASHWATI ROY<sup>a</sup>, SAVITA KHANNA<sup>b</sup>, HEILAINE M. ALESSIO<sup>b</sup>, JELENA VIDER<sup>c</sup>, DEBASIS BAGCHI<sup>c</sup>,  
MANASHI BAGCHI<sup>c</sup> and CHANDAN K. SEN<sup>a,\*</sup>

<sup>a</sup>Laboratory of Molecular Medicine, Department of Surgery, 512 Heart and Lung Research Institute, The Ohio State University Medical Center, 473 W. 12th Avenue, Columbus, OH 43210, USA; <sup>b</sup>Department of Physical Education, Health and Sport Studies, Miami University, Oxford, OH 45056, USA; <sup>c</sup>School of Pharmacy and Allied Health Professions, Creighton University, Omaha, NE 68178, USA

Accepted by Professor E. Niki

(Received 21 January 2002; in revised form 12 March 2002)

Recent studies show that edible berries may have potent chemopreventive properties. Anti-angiogenic approaches to prevent and treat cancer represent a priority area in investigative tumor biology. Vascular endothelial growth factor (VEGF) plays a crucial role for the vascularization of tumors. The vasculature in adult skin remains normally quiescent. However, skin retains the capacity for brisk initiation of angiogenesis during inflammatory skin diseases such as psoriasis and skin cancers. We sought to test the effects of multiple berry extracts on inducible VEGF expression by human HaCaT keratinocytes. Six berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seed, and strawberry) and a grape seed proanthocyanidin extract (GSPE) were studied. The extracts and uptake of their constituents by HaCaT were studied using a multi-channel HPLC-CoulArray approach. Antioxidant activity of the extracts was determined by ORAC. Cranberry, elderberry and raspberry seed samples were observed to possess comparable ORAC values. The antioxidant capacity of these samples was significantly lower than that of the other samples studied. The ORAC values of strawberry powder and GSPE were higher than cranberry, elderberry or raspberry seed but significantly lower than the other samples studied. Wild bilberry and blueberry extracts possessed the highest ORAC values. Each of the berry samples studied significantly inhibited both H<sub>2</sub>O<sub>2</sub> as well as TNF $\alpha$  induced VEGF expression by the human keratinocytes. This effect was not shared by other antioxidants such as  $\alpha$ -tocopherol or GSPE but was commonly shared by pure flavonoids. Matrigel assay using human dermal microvascular endothelial cells showed that edible berries impair angiogenesis.

**Keywords:** Angiogenesis; Edible berries; VEGF; GSPE

## INTRODUCTION

Nutrition is a major tool in cancer prevention. Assessments reported in 1981,<sup>[1]</sup> and more recently in 1995,<sup>[2]</sup> consistently indicate that almost a third of all cancer events may be prevented by changes in diet. The therapeutic property of edible berries has been long known.<sup>[3]</sup> More recently, it has been observed that edible berries may have potent chemopreventive properties.<sup>[4–8]</sup> Berries are rich in anthocyanins, flavonoid glycosides, responsible for the red, violet, purple and blue color of the fruits. Dietary consumption of anthocyanin has been shown to improve overall antioxidant defense status of human plasma.<sup>[9]</sup>

Angiogenesis is a key event that feeds tumor growth and cancer metastases. Thus, anti-angiogenic approaches to prevent and treat cancer represent a priority area in investigative tumor biology.<sup>[10,11]</sup> On one hand, the search is on for specific medical drugs that would efficiently limit tumor angiogenesis.<sup>[10,11]</sup> On the other hand, diet-based approaches to limit angiogenesis are being actively explored.<sup>[4,5,12–17]</sup> Proven safety for human use is a major merit that strengthens this latter approach. While it is evident that consumption of a plant-based diet can prevent the development and progression of tumors associated with extensive neovascularization,<sup>[12]</sup> the underlying mechanisms remain unclear.

\*Corresponding author. Tel.: +1-614-247-7786. Fax: +1-614-247-7818. E-mail: sen-1@medctr.osu.edu

Vascular endothelial growth factor (VEGF)/vascular permeability factor plays a crucial role for the vascularization of tumors including breast cancers. Tumors produce ample amounts of VEGF, which stimulates the proliferation and migration of endothelial cells, thereby inducing tumor vascularization by a paracrine mechanism. VEGF receptors are highly expressed by the endothelial cells in tumor blood vessels. VEGF expression can be induced in various cell types by a number of stimuli including cytokines and oxidants that are present at the tumor site.<sup>[18–20]</sup>

The vasculature in adult skin remains normally quiescent, due to the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. However, skin retains the capacity for brisk initiation of angiogenesis, the growth of new blood vessels from preexisting vessels, during inflammatory skin diseases such as psoriasis and skin cancers such as cutaneous squamous cell carcinomas. Moreover, cyclic vascular expansion occurs during the growth phase of the hair follicle. Recent evidence suggests VEGF as the major skin angiogenic factor.<sup>[21]</sup> During skin angiogenesis, expression of VEGF is induced in epidermal keratinocytes. VEGF is a marker of tumor invasion and metastasis in squamous cell carcinomas.<sup>[22]</sup> We sought to test the effects of multiple nutritional berry extracts on inducible VEGF expression by human keratinocytes.

## MATERIALS AND METHODS

### Materials

The berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seed and strawberry), two berry extract mixes (Mix 1-optiBerry IH141, Mix 2-optiBerry IH151; two different blends of wild blueberry, strawberry, cranberry, raspberry seed, elderberry and wild bilberry samples) and a grape seed proanthocyanidin extract (GSPE) were obtained from the InterHealth Nutraceuticals, Inc. (Benicia, CA). GSPE is a natural extract containing approximately 54% dimeric, 13% trimeric and 7% tetrameric proanthocyanidins, a small amount of monomeric bioflavonoids.<sup>[23]</sup> Unless otherwise stated all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

### High Performance Liquid Chromatography Multi-channel Electrochemical Analyses

#### Preparation of Berry Extracts for HPLC Analysis

The material was weighed (10 mg) and dissolved in 400  $\mu$ l aqueous methanol (62.5  $\pm$  0.29% BHA). Samples were ultrasonicated on ice for 2 min (30 s  $\times$  4 pulse) and 100  $\mu$ l of 6N HCl was added to

the samples. The samples were bubbled with nitrogen for 30 s and incubated at 90°C for 2 h. Samples were cooled and 500  $\mu$ l 100% methanol was added. Samples were centrifuged at 13,000 rpm for 5 min at 4°C and then filtered using a 0.45 micron filter.

### Cellular Uptake of Berry Constituents

HaCaT cells were cultured in 150 mm  $\times$  20 mm plates. After 24 h of seeding, growth media was changed to serum-free RPMI and berry extracts were added in excess (250  $\mu$ g/ml) to allow for detection of constituents taken up by cells in trace amounts. Experiments testing the effects of berry extracts on inducible VEGF expression have used a maximum of 50  $\mu$ g/ml of berry extract. A five-fold excess of the extracts were used to study cellular uptake to ensure that analytical limitations did not prevent us from detecting the presence of certain berry constituents that were taken up in low amounts. After 24 h of such treatment, cells were washed with PBS, scrapped and collected. Phosphate buffer was added to cell pellets and pellets were homogenized on wet-ice and then ultrasonicated. HCl (3 M) was added to the samples and the resulting products were incubated for 30 min at room temperature in the dark. Polyphenols were extracted with 2 ml of ethyl acetate and analyzed by coulometric electrochemical array detection with HPLC as indicated (ESA Inc., Chelmsford, MA).

### HPLC-CoulArray Detection of Flavonoid/proanthocyanidins

The gradient analytical system consisted of two pumps, an autosampler, a thermostatic chamber and a 12-channel CoulArray detector. The chromatography conditions are listed below:

- Column: Symmetry C18 5  $\mu$ m (4.6 mm  $\times$  250 mm)
- Mobile phase A: 50 mM Sodium phosphate buffer; pH 3; methanol (99:1 v/v)
- Mobile phase B: 100 mM Sodium phosphate buffer pH 3.45; acetonitrile; methanol (30:60:10 v/v/v)
- Gradient: Conditions: 0% B for 5 min to 80% B in 40 min; held at 80% B until 45 min then back to 0% B by 55 min
- Flow Rate: 0.8 ml/min
- Detector: Model 5600A, CoulArray (ESA Inc., Chelmsford, MA)
- Applied potentials:
  - i) – 20 to +100 mV in +80 mV increments
  - ii) + 160 to +400 mV in 60 mV increments
  - iii) + 500 to +700 mV in 100 mV increments.

### Cells and Cell Culture

Immortalized HaCaT human keratinocytes, kindly provided to us by Dr N. Fusenig,<sup>[24]</sup> were

grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Berry extracts and GSPE stock solutions for cell treatment were prepared fresh in dimethyl sulfoxide at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1%. Prior to cell treatment, the DMSO solutions were passed through a 0.22 µm filter for sterilization. Respective controls were treated with equal volume of dimethyl sulfoxide. HaCaT cells were pretreated with the berry samples as indicated in the respective figure legends. Treatment of cells with berry samples up to 50 µg/ml did not influence cell viability as detected by a standard lactate dehydrogenase dependent viability assay (data not shown). However, at 25 µg/ml, GSPE was toxic to cells. Following incubation with the respective berry samples, the cells were washed with serum-free medium and then treated with TNFα (25 ng/ml) or H<sub>2</sub>O<sub>2</sub> (250 µM) in a

serum-free medium as indicated in the respective figure legends.

#### Cell Viability Assay

HaCaT cells were seeded at  $0.15 \times 10^6$  cells/well/ml in 12-well plates. After 24 h of seeding, growth media was changed to serum-free RPMI and berry samples were added at a high dose (50 µg/ml). After 24 h, media was collected and centrifuged at 3500 rpm for 5 min at 4°C. Aliquots were transferred to a 96-well and lactate dehydrogenase (LDH) assay was performed using *in vitro* toxicology assay kit obtained from Sigma Chemical Co. (St. Louis, MO, USA).

#### Determination of Oxygen Radical Absorbing Capacity (ORAC)

Individual berry samples (25 mg) were dissolved in 1 ml methanol. Then, 0.09 ml phosphate buffer was added to 0.01 ml of the methanol solution obtained

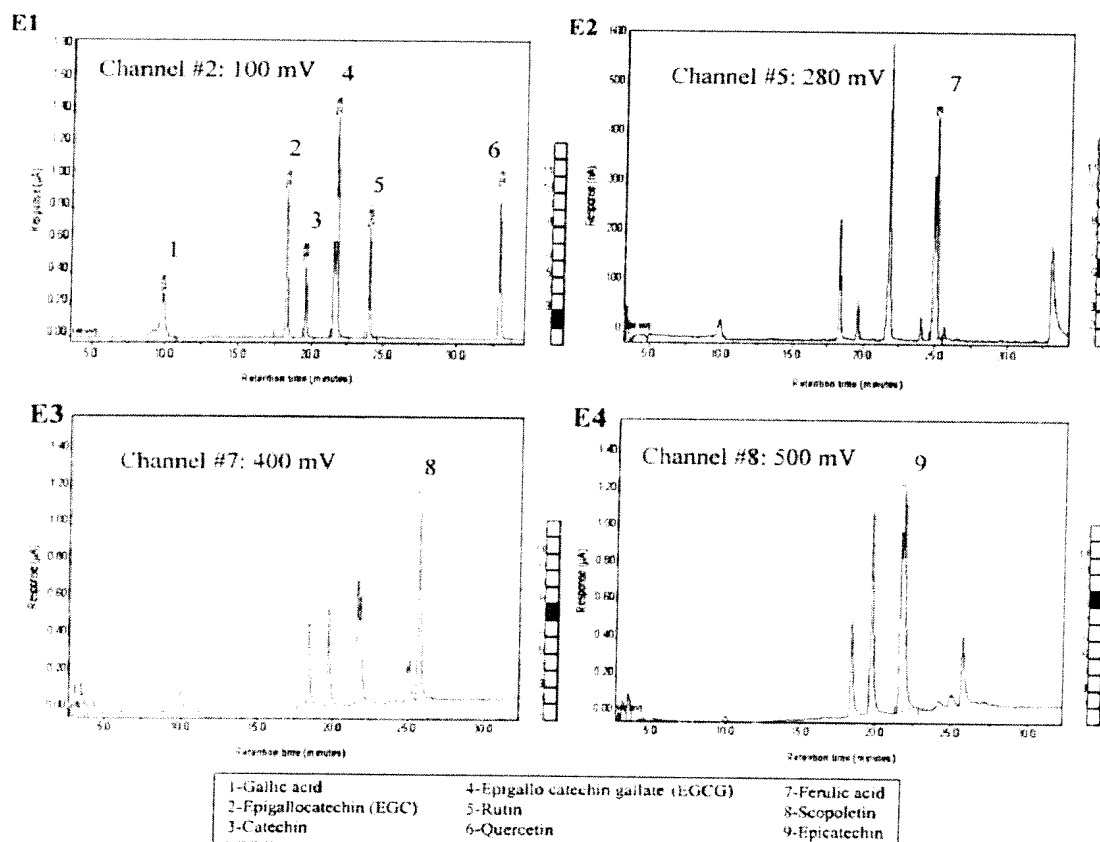


FIGURE 1. Chromatogram showing peaks of authentic standards.

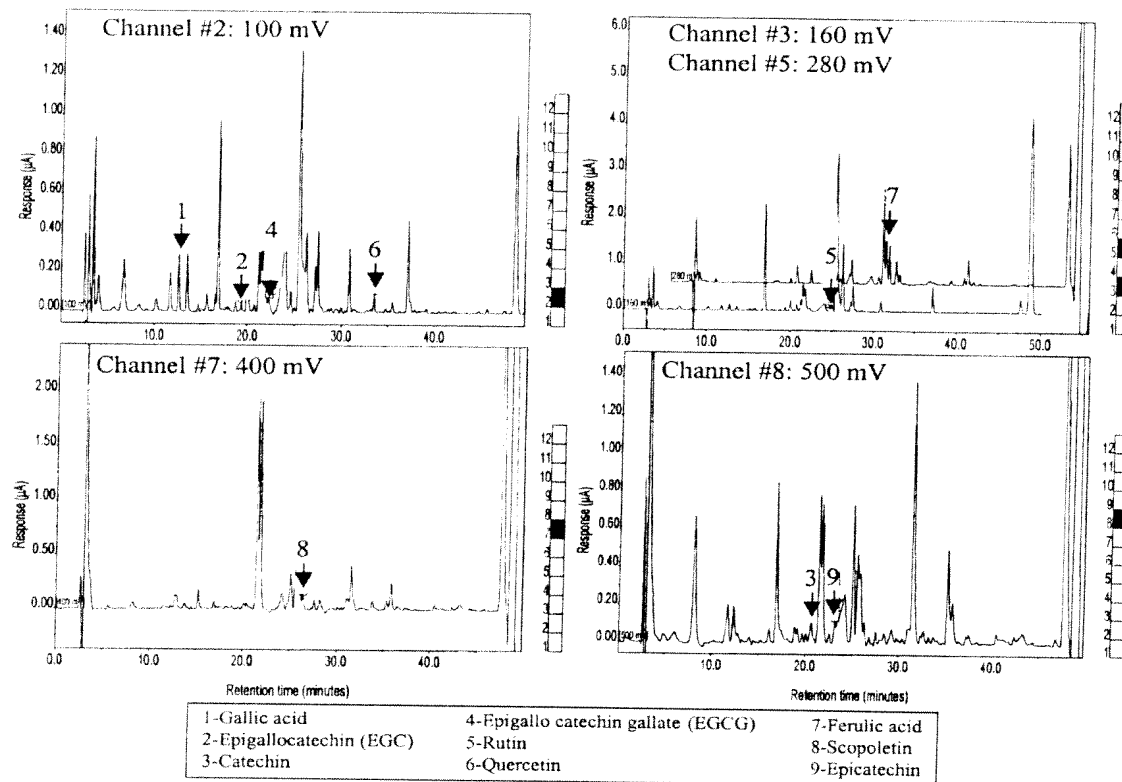


FIGURE 2. Representative CoulArray chromatograms of berry extracts showing peaks corresponding to authentic flavonoid/proanthocyanidins. Berry samples were weighed (10 mg) and dissolved in 400  $\mu$ l of mix containing 62.5% methanol and 0.29% BHA. Samples were ultrasonicated on ice for 2 min. After that 100  $\mu$ l of 6N HCl was added to the samples. Samples were bubbled with nitrogen for 30 s and incubated at 90°C for 2 h. Samples were cooled and 500  $\mu$ l 100% methanol was added. Samples were centrifuged at 13,000 rpm for 5 min at 4°C and then filtered. One hundred micrograms of berry sample was injected to HPLC. For HPLC-electrochemical detection protocol see "Methods" section. Sample shown is berry Mix 1.

as described above. A total of 0.01 ml sample was used for analysis.

The procedure for performing ORAC assays was based on a previous report of Cao *et al.*<sup>[25]</sup> This assay measures the ability of antioxidant compounds in test materials to inhibit the decline of B-phycoerythrin (B-PE) fluorescence that is induced by a peroxy radical generator, AAPH. The reaction mixture contained 1.6 ml of 75 mM phosphate buffer (pH 7.0), 200  $\mu$ l of B-PE (3.92 mg/100 ml), 200  $\mu$ l of 320 nM AAPH, and 100  $\mu$ l of sample. Trolox, a water-soluble analogue of vitamin E, was used as a control antioxidant standard. The fluorescence of B-PE was determined and recorded every 5 min at the excitation wavelength of 540 nm and emission wavelength of 570 nm using a Turner fluorometer (Sunnyvale, CA) until the fluorescence of the last reading declined to < 5% of the first reading. The final results (ORAC value) were calculated using the

differences of areas under the quenching curves of B-PE between a blank and a sample and expressed as micromoles of Trolox equivalents (TE) per gram of fresh weight.

#### Measurement of VEGF Protein

HaCaT cells were seeded onto multiple well culture-plates. After 24 h of growth (at ~80% confluency), the cells were synchronized by culturing in serum deprived medium for 12 h. Following the synchronization, cells were treated with H<sub>2</sub>O<sub>2</sub> or TNF $\alpha$ . For berry treatment protocol see legends. As described previously,<sup>[26]</sup> the reason for selecting serum-free medium was to avoid any possible interaction between the serum components and H<sub>2</sub>O<sub>2</sub>. VEGF level in the medium was determined using commercially available ELISA kit (R and D systems, Minneapolis, MN).

TABLE 1 Profile of analyzed constituents in individual berry samples

Flavonoids	Elderberry ( $\mu\text{mol}/\text{mg}$ )	Wild bilberry ( $\mu\text{mol}/\text{mg}$ )	Wild blackberry ( $\mu\text{mol}/\text{mg}$ )	Strawberry ( $\mu\text{mol}/\text{mg}$ )	Cranberry ( $\mu\text{mol}/\text{mg}$ )	Raspberry ( $\mu\text{mol}/\text{mg}$ )	Berry Mix 1 ( $\mu\text{mol}/\text{mg}$ )	Berry Mix 2 ( $\mu\text{mol}/\text{mg}$ )
Gallic acid	175.9	21.1	252.1	1183.7	134.5	343.9	571.6	273.8
Epigallocatechin	17.4	41.2	181.5	157.1	ND	20.1	61	38
Catechin	753.3	190.8	238.3	200.3	251.4	770	188.6	225.2
Epigallocatechin gallate	ND	78.9	420.9	8.3	ND	1614	15.6	512.8
Epicatechin	34.2	17.4	141.4	31.8	235.7	1140	99.2	112.4
Kumm	69.3	966.4	93.9	409.9	125.8	17364	414.4	611.4
Ferulic acid	142.7	514.5	2560.2	338.5	104.2	12130	1807	2247.4
Scopolletin	48.3	1676.4	188.7	129.7	133.2	359	149.8	191.6
Quercetin	385.8	932	65.2	6.27	63.6	17.8	89.6	144.6

Sample preparation and injection as described in legend of Fig. 2. Raspberry refers to raspberry seed powder. ND, not detected.

### *In Vitro* Angiogenesis Assay

An *in vitro* angiogenesis kit provided by CHEMICON International, Inc. (Temecula, CA) was used for the assay. In brief, the ECMatrix™ (10 × ) solution was thawed on ice and diluted with a diluent provided with the kit. ECMatrix™ is a solid gel of basement proteins prepared from the Engelbreth-Holm-Swarm (EHS) mouse tumor. The diluted ECMatrix™ (10 × ), solution (100  $\mu\text{l}$ ) was transferred to each well of a 96-well tissue culture plate and incubated at 37°C for at least 1 h to allow the matrix solution to solidify. HMVE cells are harvested and resuspended in media in the presence or absence of berry sample as shown in Fig. 7. Cells (5000 cells/well) were added on top of the solidified matrix solution and maintained in a cell culture incubator at 37°C overnight. Endothelial tube formation was observed and digitally photographed under an inverted light microscope at 20 × magnification.

## RESULTS

### Analysis of Berry Constituents and Cellular Uptake

The coulometric electrochemical array detection with HPLC offers several advantages over LC/UV detection. In addition to having a much higher sensitivity of detection, the CoulArray™ (ESA Inc., Chelmsford, MA) detector provides on-line generation of qualitative data and the ability to resolve peaks on the basis of different voltammetric characteristics. In flavonoids and proanthocyanidins, the voltammetric characteristics are expected to be different for trihydroxy, dihydroxy or monohydroxy substituents of the phenol ring. An absorbance-based detector is unable to distinguish these chemical differences and will generate the same absorbance curve for compounds with such minor chemical differences. Figure 1 shows the simultaneous detection of nine different constituents of interest. Figure 2 shows simultaneous detection of these nine constituents in a berry sample. Results from the individual study of the composition on each berry sample are presented in Table 1. Raspberry seed powder was several-folds richer in the various constituents compared to any of the whole berry samples. The strawberry powder contained significant amounts of gallic acid while the most abundant constituents in the elderberry extract were catechin and quercetin. Overall, the cranberry powder did not contain high amounts of any of the constituents studied. Catechin and epicatechin were the two most abundant constituents detected in cranberry powder. Wild blueberry extract was primarily dominated by the presence of ferulic acid. However, the ferulic acid concentration was much lower in wild blueberry

TABLE II Cellular uptake of berry powder constituents by human keratinocytes

Flavonoids	Wild bilberry (pmol/mg)	Wild blueberry (pmol/mg)	Strawberry (pmol/mg)	Raspberry (pmol/mg)	Berry Mix 1 (pmol/mg)	Berry Mix 2 (pmol/mg)
Gallic acid	ND	ND	ND	ND	ND	ND
Epigallocatechin	16.64	58.4	28.32	24.08	17.2	34.56
Catechin	174.64	257.68	95.12	74.4	246	238.48
Epigallocatechin gallate	96.64	140	61.76	367.44	166.32	138.32
Epicatechin	47.12	24	97.68	48.48	51.52	74.48
Rutin	73.52	157.52	59.28	16.16	31.76	26.24
Ferulic acid	110.08	15.36	38.32	82.16	72.32	113.04
Scopoletin	54.88	118.16	43.04	84.88	63.92	134.08
Quercetin	27.12	23.44	31.44	8.96	6.24	9.14

HaCaT cells were cultured in 150 mm  $\times$  20 mm plates. After 24 h of growth, media was changed to serum-free RPMI and berry samples were added in excess (250  $\mu$ g/ml) to increase sensitivity of detection of trace constituents in the cell. After 24 h, cells were washed with PBS, scrapped and collected. Phosphate buffer was added to cell pellets and pellets were homogenized on wet-ice and then ultrasonicated. HCl (3M) was added and the samples were incubated for 30 min at room temperature in the dark. Polyphenols were extracted with 2 ml of ethyl acetate and analyzed by coulometric electrochemical array detection with HPLC. Berry Mix 1, IH141; Berry Mix 2, IH151 (Inter Health Nutraceuticals, Benicia, CA). Raspberry refers to raspberry seed powder. ND, not detected.

extract compared to that in raspberry seed powder. Among all the whole berry samples studied, epigallocatechin gallate was most abundant in wild blueberry extract. The most abundant constituents of wild bilberry extract were scopoletin, rutin, quercetin and ferulic acid. The respective compositions of each of the two berry mixtures IH141 and IH151 are shown in Table I.

To test the uptake of the individual constituents by human keratinocytes, HaCaT cells were treated with each of the two berry mixtures as indicated in the legend of Table II. Cells were then washed and processed for HPLC analysis. Comparing Tables I and II it may be observed that the original composition profile of the berry mixture and the profile of constituents present in the cell do not match. The cells preferably took up catechin and epigallocatechin gallate. We also attempted to study the cellular uptake profile of GSPE. At 25  $\mu$ g/ml and beyond, GSPE proved to be toxic to cells (not shown) and the uptake study could not be performed. The berry samples, however, did not exhibit any toxicity (data not shown).

#### Antioxidant Capacity as Detected by ORAC

The peroxyl-radical scavenging capacity of the berry samples and GSPE was studied using the ORAC assay commonly used to study antioxidant capacity of herbal extracts.<sup>[27–33]</sup> Cranberry, elderberry and raspberry seed samples were observed to possess comparable ORAC values. The antioxidant capacity of these samples was significantly lower than that of the other samples studied. The ORAC values of strawberry powder and GSPE were higher than cranberry, elderberry or raspberry seed but significantly lower than the other samples studied. Wild bilberry and blueberry extracts possessed the highest ORAC values. These values were compar-

able to the ORAC values of the two berry mixtures (Fig. 3).

#### Anti-angiogenic Properties

First, the effect of these berry samples on inducible VEGF expression by HaCaT cells was investigated. Figures 4 and 5 show that each of the berry samples studied potently inhibited both H<sub>2</sub>O<sub>2</sub> as well as TNF $\alpha$  induced VEGF expression by the human keratinocytes. Our next goal was to test whether other antioxidants shared this property of the berry extract. Antioxidants such as GSPE, with comparable ORAC (Fig. 3), or  $\alpha$ -tocopherol (Fig. 6) did not influence inducible VEGF expression suggesting that the observed effect of berry samples was not

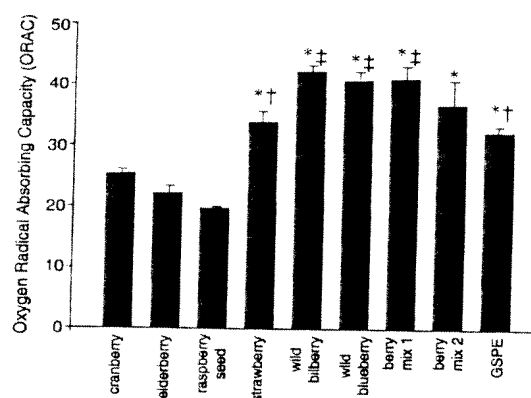


FIGURE 3 Antioxidant activity of berry samples. ORAC assays for berry samples were carried out following method as described in Experimental Protocols. Final results (ORAC value) were calculated and expressed using Trolox equivalents per gram weight basis. GSPE: grape seed proanthocyanidin extract.  $^*p < 0.05$ ;  $^{\dagger}$ , higher compared to cranberry, elderberry and raspberry seed;  $^{\ddagger}$ , lower compared to bilberry, blueberry or mix 1;  $^{\S}$ , higher than all other samples. Mean  $\pm$  SD of three experiments.

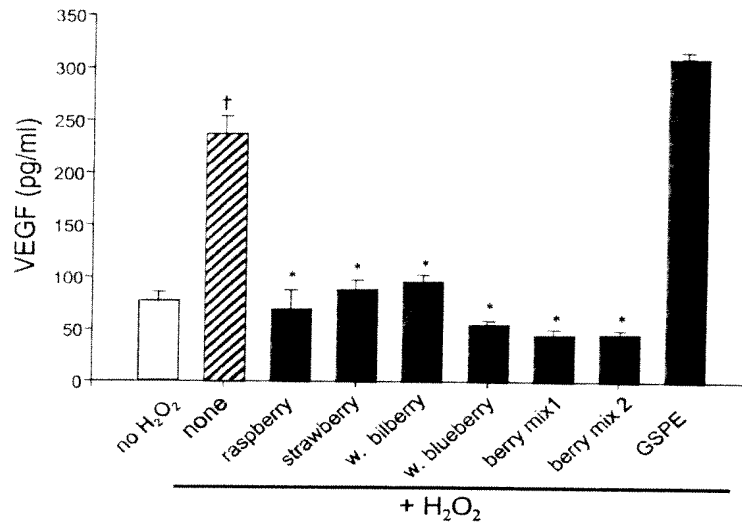


FIGURE 4 Berry samples inhibit oxidant-induced expression of VEGF. HaCaT cells were seeded at density  $0.45 \times 10^6$ /well/3 ml. After 24 h, growth media was changed to serum-free RPMI and berry samples (50  $\mu$ g/ml) were added. After 12 h, cells were challenged with H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M). After 12 h of activation with H<sub>2</sub>O<sub>2</sub>, media was collected for ELISA. \*<sup>†</sup>,  $p < 0.05$ . <sup>†</sup>, higher in response to H<sub>2</sub>O<sub>2</sub> treatment; \*, lower compared to H<sub>2</sub>O<sub>2</sub> treated cells. Mean  $\pm$  SD of three experiments.

dependent on their antioxidant property. Of importance, pure flavonoids such as ferrulic acid, catechin and rutin shared the ability to suppress oxidant-inducible VEGF expression (Fig. 6). Thus, it was evident that the flavonoid component of the berry samples may have been responsible for the observed effect on inducible VEGF expression and release. Next, we sought to test whether the berry samples

influence the process of angiogenesis *per se*. Among the various *in vivo* and *in vitro* methods for the study of angiogenesis, the *in vitro* matrigel assay represents a highly reliable approach to test angiogenic or antiangiogenic properties of test species.<sup>[34]</sup> The method is based on the differentiation of endothelial cells to form capillary like structures on a basement membrane matrix, Matrigel, derived from EHS

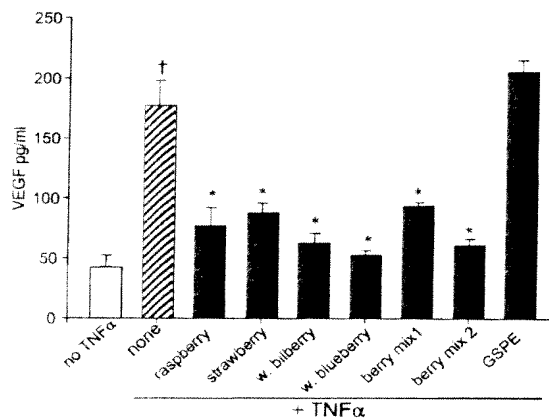


FIGURE 5 Berry samples inhibit TNF $\alpha$ -induced expression of VEGF. HaCaT cells were seeded at density  $0.45 \times 10^6$ /well/3 ml. After 24 h, growth media was changed to serum-free RPMI and berry samples (50  $\mu$ g/ml) were added. After 12 h, cells were challenged with TNF $\alpha$  (25 ng/ml). After 12 h of activation with TNF $\alpha$ , media was collected for ELISA. \*<sup>†</sup>,  $p < 0.05$ . <sup>†</sup>, higher in response to TNF $\alpha$  treatment; \*, lower compared to TNF $\alpha$  treated cells. Mean  $\pm$  SD of three experiments.

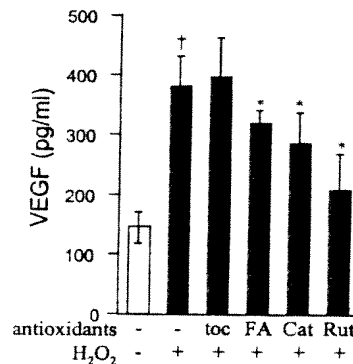


FIGURE 6 Effect of pure flavonoid and tocopherol on oxidant-induced VEGF expression. HaCaT cells were seeded at density  $0.45 \times 10^6$ /well/3 ml. After 24 h, growth media was changed to serum-free RPMI and either pure flavonoids (ferrulic acid, FA 200  $\mu$ M; catechin, Cat 100  $\mu$ M; rutin, rut 1  $\mu$ M) at concentrations observed in berry samples (see Fig. 3) or tocopherol (10  $\mu$ M, as a reference antioxidant) were added. After 12 h, cells were challenged with H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M). After 12 h of activation with H<sub>2</sub>O<sub>2</sub>, media was collected for ELISA. \*<sup>†</sup>,  $p < 0.05$ . <sup>†</sup>, higher in response to H<sub>2</sub>O<sub>2</sub> treatment; \*, lower compared to H<sub>2</sub>O<sub>2</sub> treated cells. Mean  $\pm$  SD of three experiments.





FIGURE 7 Anti-angiogenic property of berry samples *in vitro*. The test was conducted in an *in vitro* model of angiogenesis using Matrigel and human microvascular endothelial cells. For the assay, human microvascular endothelial cells  $0.5 \times 10^5$  cells/per well were seeded onto 4-well plates precoated with Matrigel. After 48 h of seeding, berry samples were added ( $50 \mu\text{g}/\text{ml}$ ). Endothelial tube formation is observed and digitally photographed under an inverted light microscope at  $20\text{--}100\times$  ( $20\times$  shown) magnification. Representative of three experiments.

tumor. Matrigel is a matrix of a mouse basement membrane neoplasm. It represents a complex mixture of basement membrane proteins including laminin, type IV collagen, entactin/nitrogen and proteoglycan sulfate, and also contains growth factors. Matrigel induces endothelial cells to differentiate as evidenced by both the morphologic changes and by the reduction in proliferation and, therefore, offers a convenient and reliable model to study biochemical and molecular events associated with angiogenesis. We used human dermal microvascular endothelial cells for this assay that was performed using a kit where the conditions are optimized for maximal capillary-like structure formation. Under basal conditions as specified by the kit, we were able to obtain numerous long capillary-like structures (Fig. 7). Treatment of the human endothelial cells with either berry Mix 1 or 2 impaired *in vitro* angiogenesis (Fig. 7).

## DISCUSSION

Anti-angiogenic properties of edible plant products have been previously reported.<sup>[13,15]</sup> Flavonoids, sulphated carbohydrates, or triterpenoids have been suspected to be the active anti-angiogenic components of plant products.<sup>[17]</sup> Catechins and polyphenols from plant extracts such as green tea show potent anticancer activity.<sup>[4]</sup> Silymarin, a naturally occurring flavonoid antioxidant, exhibits anti-cancer effects against several epithelial cancers.<sup>[16]</sup> It has been proposed that flavonoids may contribute to the preventive effect of a plant-based diet on chronic diseases, including solid tumor.<sup>[12]</sup> Although there is a general agreement that certain plant products may possess anti-angiogenic properties, the underlying mechanisms are not well characterized. A recent report has shown that resveratrol, a phytoalexin found in grapes, berries, and peanuts, is one of the most promising agents for cancer prevention. It was observed that the antitumor activity of resveratrol occurs through p53-mediated apoptosis. Both ERKs

and p38 kinase mediated resveratrol-induced activation of p53 and apoptosis through phosphorylation of p53 at serine 15.<sup>[7]</sup> Another recent paper investigated the mechanisms underlying cancer chemopreventive properties of berries. It was observed that berry extracts inhibit cellular transformation.<sup>[8]</sup> The current work presents first evidence showing that berry extracts potentially inhibit inducible VEGF expression. Some antioxidants have been observed to have anti-angiogenic effects.<sup>[35]</sup>

However, our observation that GSPE possessing high antioxidant capacity failed to inhibit inducible VEGF expression suggests that the antioxidant property alone may not account for the observed effect. This contention is consistent with the findings that numerous plant-product constituents serve as potent regulator of several signal transduction pathways.<sup>[4,7,36,37]</sup> Our results with pure monomeric flavonoids present first evidence that flavonoids may serve as potent inhibitors of inducible VEGF expression and that the flavonoid content of the berry extracts may have been responsible for the observed effect. According to manufacturers disclosure, monomeric flavonoids account for less than 1% of GSPE and this may explain the observed inability of GSPE to inhibit inducible VEGF expression. In addition to their inhibitory effect on inducible VEGF expression, berry extract impaired angiogenesis *in vitro* suggesting that other key events in angiogenesis such as integrin function<sup>[38]</sup> may be sensitive to berry constituents. These observations provide a firm mechanism-based support to the contention that edible berries may provide a feasible diet-based approach to prevent the angiogenesis-related disorders such as cancer and inflammation.<sup>[5,7,8,39]</sup>

## Acknowledgements

Supported in part by NIH GM 27345 to CKS. The Laboratory of Molecular Medicine is the research division of the Center of Minimally Invasive Surgery.

## References

- [1] Doll, R. and Peto, R. (1981) "The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today", *J. Natl Cancer Inst.* **66**, 1191–1308.
- [2] Willett, W.C. (1995) "Diet, nutrition, and avoidable cancer", *Environ. Health Perspect.* **103**(Suppl. 8), 165–170.
- [3] Otek, L., Goldhar, J., Zatriri, D., Lis, H., Adar, R. and Sharon, N. (1991) "Anti-*Escherichia coli* adhesion activity of cranberry and blueberry juices", *N. Engl. J. Med.* **324**, 1599.
- [4] Colic, M. and Pavelic, K. (2000) "Molecular mechanisms of anticancer activity of natural dietetic products", *J. Mol. Med.* **78**, 333–336.
- [5] Krestiv, L.A., Morse, M.A., Morgan, C., Carlton, P.S., Lu, J., Gupta, A., Blackwood, M. and Stoner, G.D. (2001) "Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries", *Cancer Res.* **61**, 6112–6119.
- [6] McCarty, M.F. (2001) "Current prospects for controlling cancer growth with non-cytotoxic agents—nutrients phytochemicals, herbal extracts, and available drugs", *Med. Hypotheses* **56**, 137–154.
- [7] She, Q.B., Bode, A.M., Ma, W.Y., Chen, N.Y. and Dong, Z. (2001) "Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase", *Cancer Res.* **61**, 1604–1610.
- [8] Xue, H., Aziz, R.M., Sun, N., Cassady, J.M., Kamendulis, L.M., Xu, Y., Stoner, G.D. and Klaunig, J.E. (2001) "Inhibition of cellular transformation by berry extracts", *Carcinogenesis* **22**, 351–356, Erratum appears in *Carcinogenesis* 2001 May; **22**(5), 831–833.
- [9] Cao, G. and Prior, R.L. (1999) "Anthocyanins are detected in human plasma after oral administration of an elderberry extract", *Clin. Chem.* **45**, 574–576.
- [10] Giavazzi, R. and Taraboletti, G. (1999) "Angiogenesis and angiogenesis inhibitors in cancer", *Forum* **9**, 261–272.
- [11] Griffiths, A.W. and Molema, G. (2000) "Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation", *Pharmacol. Rev.* **52**, 237–268.
- [12] Fotis, T., Pepper, M.S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wahala, K., Montesano, R. and Schweigere, L. (1997) "Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis", *Cancer Res.* **57**, 2916–2921.
- [13] Fotis, T., Pepper, M.S., Montesano, R., Aktas, E., Breit, S., Schweigere, L., Rasku, S., Wahala, K. and Adlercreutz, H. (1998) "Phytoestrogens and inhibition of angiogenesis", *Baillière's Clin. Endocrinol. Metab.* **12**, 649–666.
- [14] Hayashi, A., Gillen, A.C. and Lott, J.R. (2000) "Effects of daily oral administration of quercetin chalcone and modified citrus pectin", *Altern. Med. Rev.* **5**, 546–552.
- [15] Hisa, T., Kimura, Y., Takada, K., Suzuki, F. and Takigawa, M. (1998) "Shikonin, an ingredient of *Lithospermum erythrorhizon*, inhibits angiogenesis *in vivo* and *in vitro*", *Anticancer Res.* **18**, 783–790.
- [16] Jiang, C., Agarwal, R. and Lu, J. (2000) "Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells", *Biochem. Biophys. Res. Commun.* **276**, 371–378.
- [17] Paper, D.H. (1998) "Natural products as angiogenesis inhibitors", *Planta Med.* **64**, 686–695.
- [18] Chandel, N.S., Trzyna, W.C., McClintock, D.S. and Schumacker, P.T. (2000) "Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin", *J. Immunol.* **165**, 1013–1021.
- [19] Schwartzburg, P.M. and Lankin, V.Z. (1995) "Endogenous induction of transient oxidant-imbalances in Ehrlich cells as a possible trigger to fast tumor fluid accumulation", *Med. Oncol.* **12**, 203–207.
- [20] Lee, F.Y., Li, Y., Zhu, H., Yang, S., Lin, H.Z., Trush, M. and Diehl, A.M. (1999) "Tumor necrosis factor increases mitochondrial oxidant production and induces expression of uncoupling protein-2 in the regenerating mice [correction of rat] liver", *Hepatology* **29**, 677–687.
- [21] Detmar, M. (2000) "The role of VEGF and thrombospondins in skin angiogenesis", *J. Dermatol. Sci.* **24**(Suppl. 1), S78–S84.
- [22] Sauter, E.R., Nesbit, M., Watson, J.C., Klein-Szanto, A., Litwin, S. and Herlyn, M. (1999) "Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck", *Clin. Cancer Res.* **5**, 775–782.
- [23] Joshi, S.S., Kuszyński, C.A., Benner, E.J., Bagchi, M. and Bagchi, D. (1999) "Amelioration of the cytotoxic effects of chemotherapeutic agents by grape seed proanthocyanidin extract", *Antioxid. Redox Signal.* **1**, 563–570.
- [24] Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N.E. (1988) "Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line", *J. Cell Biol.* **106**, 761–771.
- [25] Cao, G., Alessio, H.M. and Cutler, R.G. (1993) "Oxygen-radical absorbance capacity assay for antioxidants", *Free Radic. Biol. Med.* **14**, 303–311, [See comments].
- [26] Khanna, S., Roy, S., Bagchi, D., Bagchi, M. and Sen, C.K. (2001) "Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract", *Free Radic. Biol. Med.* **31**, 38–42.
- [27] Caldwell, C.R. (2001) "Oxygen radical absorbance capacity of the phenolic compounds in plant extracts fractionated by high-performance liquid chromatography", *Anal. Biochem.* **293**, 232–238.
- [28] Wang, S.Y. and Stretch, A.W. (2001) "Antioxidant capacity in cranberry is influenced by cultivar and storage temperature", *J. Agric. Food Chem.* **49**, 969–974.
- [29] Jiao, H. and Wang, S.Y. (2000) "Correlation of antioxidant capacities to oxygen radical scavenging enzyme activities in blackberry", *J. Agric. Food Chem.* **48**, 5672–5676.
- [30] Prior, R.L. and Cao, G. (2000) "Analysis of botanicals and dietary supplements for antioxidant capacity: a review", *J. AOAC Int.* **83**, 950–956.
- [31] Lee, C. (2000) "Antioxidant ability of caffeine and its metabolites based on the study of oxygen radical absorbing capacity and inhibition of LDL peroxidation", *Clin. Chim. Acta* **295**, 141–154.
- [32] Wang, S.Y. and Lin, H.S. (2000) "Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage", *J. Agric. Food Chem.* **48**, 140–146.
- [33] Prior, R.L. and Cao, G. (1999) "Antioxidant capacity and polyphenolic components of teas: implications for altering *in vivo* antioxidant status", *Proc. Soc. Exp. Biol. Med.* **220**, 255–261.
- [34] Ponce, M.L., Nomizu, M. and Kleinman, H.K. (2001) "An angiogenic laminin site and its antagonist bind through the alpha5beta3 and alpha5beta1 integrins", *FASEB J.* **15**, 1389–1397.
- [35] Nishimura, G., Yanoma, S., Mizuno, H., Kawakami, K. and Tsukuda, M. (1999) "An antioxidant, probucol, induces anti-angiogenesis and apoptosis in athymic nude mouse xenografted human head and neck squamous carcinoma cells", *Jpn J. Cancer Res.* **90**, 1224–1230.
- [36] Wiseman, S., Mulder, T. and Rietveld, A. (2001) "Tea flavonoids: bioavailability *in vivo* and effects on cell signaling pathways *in vitro*", *Antioxid. Redox Signal.* **3**, 1009–1021.
- [37] Rice-Evans, C. and Bagchi, D. (2001) "Nutritional proanthocyanidins, flavonoids and related phenols", *Antioxid. Redox Signal.* **3**, 939–940.
- [38] Maeshima, Y., Yerramalla, U.L., Dhanabal, M., Holthaus, K.A., Barbashov, S., Kharbanda, S., Reimer, C., Manfredi, M., Dickerson, W.M. and Kalluri, R. (2001) "Extracellular matrix-derived peptide binds to alpha5beta3 integrin and inhibits angiogenesis", *J. Biol. Chem.* **276**, 31959–31968.
- [39] Xia, Z.Q., Costa, M.A., Pelissier, H.C., Davin, L.B. and Lewis, N.G. (2001) "Secoisolaricresinol dehydrogenase purification, cloning, and functional expression: Implications for human health protection", *J. Biol. Chem.* **276**, 12614–12623.